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Development of reagents to study the turkey's immune response

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journal homepage: www.elsevier.com/locate/dciDevelopment of reagents to study the turkey's immune response: Identification and molecular cloning of turkey CD4, CD8 α and CD28Fiona Powell¹, Michelle Lawson², Lisa Rothwell, Pete Kaiser^{*}

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ABSTRACT

The cDNAs of three turkey CD markers, CD4, CD8 α and CD28, were identified by screening a turkey cDNA library. The coding regions of the chicken and turkey genes are highly conserved, with 91.3–96.1% nucleotide (nt) and 84.2–95.5% amino acid (aa) identity. Identity was less conserved between avian CD markers and their mammalian homologues, ranging from 44.7 to 59.8% and 22.4 to 50.4% at the nt and aa levels, respectively. Anti-chicken CD8 α and CD28 monoclonal antibodies were demonstrated to specifically cross-react with turkey CD8 α and CD28, respectively.

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1. Introduction

The greater economical importance of the chicken has led to more extensive identification of immune-related molecules in this species than in any other avian species, including the turkey. As a result, a wide and ever increasing range of reagents are available to measure the chicken's immune response. This 'immunological toolbox' includes monoclonal antibodies (mAb) raised against a number of cell surface markers in the chicken, including CD4, CD8 α and CD28, which have long enabled investigation of immune cell subsets during infection studies. The vast majority of these markers have yet to be cloned in the turkey.

CD4 is a surface glycoprotein principally expressed as a monomer on T cells and thymocytes but also on macrophages and dendritic cells. CD4 acts as a co-receptor for MHC class II through interactions with the MHC class II α 2 and β 2 domains [1,2], thus restricting Th cells to recognition of antigen presented by MHC class II. Chicken CD4 was first identified using mAb produced against chicken T cells, and observing the distribution of staining of these mAb on cells from lymphoid and haemopoietic tissues [3]. Chicken CD4 is expressed on the surface of

thymocytes, peripheral T cells and intestinal intraepithelial T cells [3] and shares a similar Ig-like structure to mammalian CD4 with four extracellular Ig domains that resemble the C and V regions of Ig [4]. With similar functions to mammalian CD4, chicken CD4 therefore plays a crucial role in the immune response [5].

CD8 α is a surface glycoprotein expressed on cytotoxic T cells (Tc) as a homodimer (CD8 $\alpha\alpha$) or heterodimer (CD8 $\alpha\beta$). Expression of the β chain requires co-expression of the α chain [6]. CD8 is a co-receptor for MHC class I and, in a similar manner to CD4, restricts CD8 $^{+}$ cells to recognition of antigen presented by MHC class I. CD8 binds to the α 3 domain of the MHC molecule, enhancing the interaction between the Tc cell and the antigen presenting cell (APC). As with chicken CD4, chicken CD8 α was first identified using mAb produced against T cells [3]. In mammals and the chicken, CD8 $\alpha\alpha$ is expressed on thymocytes, peripheral T cells, NK cells, intestinal intraepithelial cells, and a population of peripheral blood CD4 T cells. CD8 $\alpha\beta$ is expressed, in mammals and the chicken, on thymocytes, peripheral T cells and in the chicken only, a subset of intestinal epithelial $\gamma\delta$ T cells [7]. As with mammals, expression of the β chain is dependent on the presence of the α chain [7].

CD28 is a surface glycoprotein expressed as a homodimer in mammals, predominantly on T cells, including naïve T cells, and provides co-stimulation to other immune effector cells. CD28 acts as a co-receptor for two further molecules found on APC, CD80 and CD86 [8]. Interactions of these molecules augment signalling from the TCR to enhance cytokine production, in particular IL-2 [9]. Chicken CD28 was first cloned from a chicken

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cDNA library by expression cloning with the mAb AV7 [10]. Expression of chicken CD28 is conserved with that of mammals, being expressed on resting and activated T cells [11]. The function of CD28 is also conserved between mammals and birds [12].

There have been several reports [13–15] using anti-chicken CD marker mAb to demonstrate cross-reactivity with turkey cells. Certain anti-chicken CD4 and CD8 α mAb cross-react with turkey cells, but not others [13,14]. More recently, we used a similar panel of mAb to follow cellular changes indicative of an inflammatory response in the footpads of growing turkeys suffering from footpad dermatitis [15]. However, demonstration of cross-reactivity does not prove that it is specific to the orthologous molecule in another species. We therefore set out to clone and sequence turkey CD4, CD8 α and CD28, and demonstrate that the cross-reactivity of the anti-chicken CD marker mAb was specific to the orthologous CD marker in the turkey.

2. Materials and methods

2.1. Turkey cDNA library screening

A normalised full-length turkey cDNA library was commercially constructed (GATC Biotech AG, Cambridge, UK) from combined turkey RNA isolated from spleen, thymus and bursa in this laboratory, so that the library contained as many immune-relevant cDNA sequences as possible. Inserts were directionally cloned into the pBluescript II SK+ vector with a modified multiple cloning site and electroporated into *E. coli* XL1-Blue MRF' cells. The cDNA library was provided as a set of three gridded filters by ARK-Genomics (Roslin Institute, Roslin, UK).

Partial length turkey cDNA clones generated in this laboratory previously [16] were labelled with $\alpha^{32}\text{P}$ -dCTP using a random-primed DNA labelling kit (Roche, Lewes, UK) and purified using Illustra ProbeQuant G-50 Micro Columns (GE Healthcare Life Sciences, Little Chalfont, UK) to remove all unincorporated labelled nucleotides from the reaction. The purified probes were used to screen the turkey cDNA library. Positive clones were isolated, purified and then sequenced with a CEQ 8000 sequencer (Beckman Coulter, High Wycombe, UK), analysed for potential turkey cytokine sequences using DS Gene (Accelrys, Cambridge, UK) and Vector NTI (Invitrogen, Paisley, UK), and compared to the relevant chicken cDNA sequences. Sequences were submitted to best local alignment search tool (BLAST) searches through the Ensembl BLAST facility (www.ensembl.org).

Sequence-specific primers were then used to generate PCR products for cloning into pCI-neo and expression in COS-7 cells.

2.2. Staining of transfected COS-7 cells

COS-7 cells were grown to confluence and used to seed 4-chamber slides (Nunc, Rochester, NY, USA) with 1×10^5 cells per chamber in a total of 1.2 ml pre-warmed COS cell growth media [17], and cultured for 24 h at 37 °C, 5% CO₂. DNA complexes were prepared in 500 μl serum-free medium with 3.8 μg DNA, 5 μl chloroquine and 3 μl DEAE/dextran. The cell layer was washed in PBS and the DNA complex added for 3 h incubation at 37 °C, 5% CO₂. DMSO was added at 10% in PBS for 2 min, removed and replaced with complete growth medium for 40 h incubation at 37 °C, 5% CO₂.

The cell layer was washed in PBS and the chambers carefully removed from the slides. The cells were fixed in acetone for 10 min, washed and blocked with 1% BSA in PBS for 1 h at room temperature, then washed three times in PBS. The relevant primary mAb, diluted 1:10 in blocking buffer, was added to the

cells with incubation for 1 h at room temperature. Cells were washed in PBS and then incubated with biotinylated mouse anti-IgG secondary antibody for 30 min. Incubation with the macro-molecular avidin-biotinylated enzyme 'ABC' conjugate (Vector Labs, Burlingame, CA, USA) followed for 30 min, prior to development with the NovaRed chromogen system (Vector Labs) to visualize peroxidase in the tissue sections. Washes with PBS took place between each step. Slides were washed in SuperQ H₂O before mounting.

3. Results

3.1. Cloning of turkey CD8 α cDNA

Screening of the turkey cDNA library identified three positive clones for CD8 α which were sequenced with plasmid-specific primers. Sequence was obtained for the full-length coding sequence plus 43 nucleotides (nt) of 5' UTR.

Sequence comparisons (Fig. 1) showed that identity between turkey CD8 α and human CD8 α was 48.5% and 29.6% at the nt and amino acid (aa) levels, respectively. Identity between turkey and mouse CD8 α was 50.7% and 26.6% at the nt and aa levels, respectively. Mammalian and avian CD8 α shared eight conserved cysteine residues.

Identity between chicken and turkey CD8 α was 92% at the nt level and 86.8% at the aa level (31/236 aa differences, Fig. 1). There was one aa insertion of a lysine at position 159 in the turkey sequence, compared to the chicken sequence, in a proline/serine/threonine-rich region previously identified in the chicken [7]. In this region there were 16/42 aa changes between the chicken and the turkey, with two serine, two proline and no threonine substitutions. Only one aa replacement was present in the transmembrane region [7]. The tyrosine protein kinase-binding site [18] was completely conserved between the chicken and turkey. The turkey CD8 α sequence was submitted to Ensembl with Acc. no. AM884251.

3.2. Cloning of turkey CD28 cDNA

Screening of the turkey cDNA library identified six positive clones for turkey CD28. Sequencing of these clones generated the whole coding sequence of turkey CD28 together with 66 nt of 5' UTR and 113 nt of 3' UTR.

Sequence comparisons (Fig. 2) showed that identity between turkey and human CD28 was 59.8% and 50.4% at the nt and aa levels, respectively. Turkey and murine CD28 shared 54.7% nt and 48.4% aa identity, respectively. Chicken and turkey CD28 showed 96.1% and 95.5% (10/221 aa differences) identity at the nt and aa levels, respectively.

The extracellular MYPPPY motif in the complementarity-determining region, CDR3, of the V-like domain was conserved between all four CD28 sequences. There were five cysteines present in the avian sequences. Four of these were present in the predicted mature proteins and were conserved in human and mouse CD28. Both chicken and turkey CD28 lacked two cysteines in their mature proteins compared to mammals. One of these cysteine residues formed an inter-molecular disulphide bond for homodimer formation in mammalian CD28. Therefore, both avian CD28 molecules could be expressed as monomers [10]. Like mammalian CD28, the extracellular regions of chicken and turkey CD28 contained seven potential N-linked glycosylation sites, compared to six in mammals, three of which were conserved in location and two in aa sequence. The turkey CD28 sequence was submitted to Ensembl with Acc. no. AM884252.

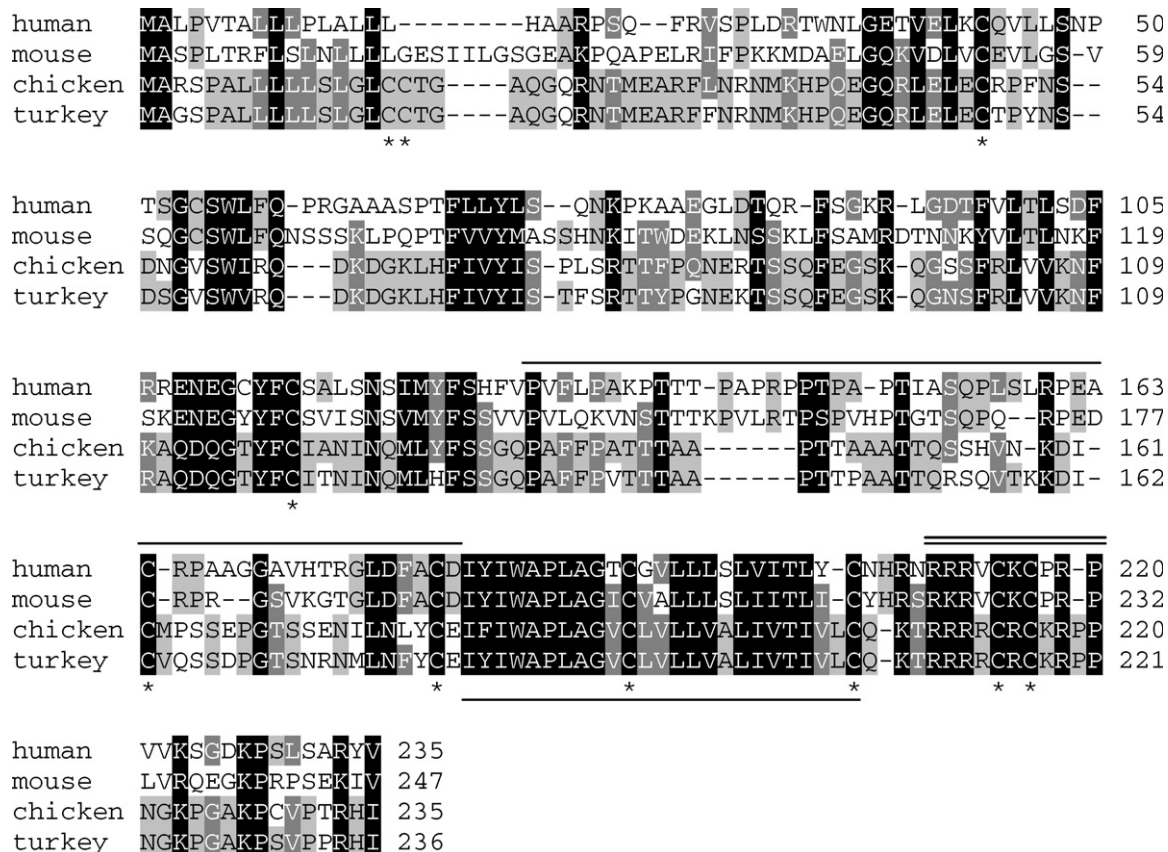


Fig. 1. Comparison of the predicted aa sequences of human, murine, chicken and turkey CD8α. Shaded areas represent conservation of aa similarity; the darker the shading, the more conserved the residue across species. Dashes indicate gaps in the alignment. Cysteine residues in the avian predicted proteins are indicated with asterisks. A proline/serine/threonine-rich region is shown overlined. The tyrosine protein kinase-binding site [18] is shown double over-lined and the transmembrane domain underlined [7]. The accession numbers of the cDNA sequences used are as follows: human, BC025715; mouse, NM_001081110; chicken, NM_205235; turkey, AM884251.

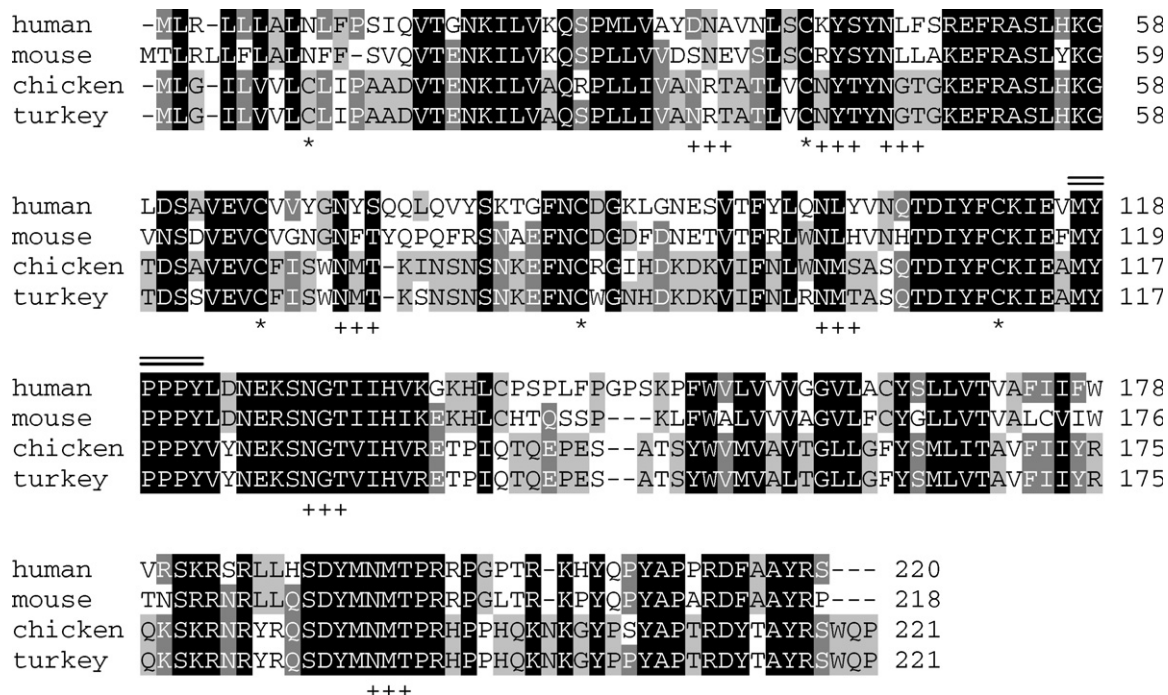


Fig. 2. Comparison of the predicted aa sequences of human, murine, chicken and turkey CD28. Shaded areas represent conservation of aa similarity; the darker the shading, the more conserved the residue across species. Dashes indicate gaps in the alignment. Conserved cysteine residues in the avian predicted proteins are indicated with asterisks. The MYPPPY motif is shown double over-lined. Potential N-linked glycosylation sites in the avian sequences are indicated by +++. The accession numbers of the cDNA sequences used are as follows: human, NM_006139; mouse, NM_007642; chicken, NM_205311; turkey, AM884252.

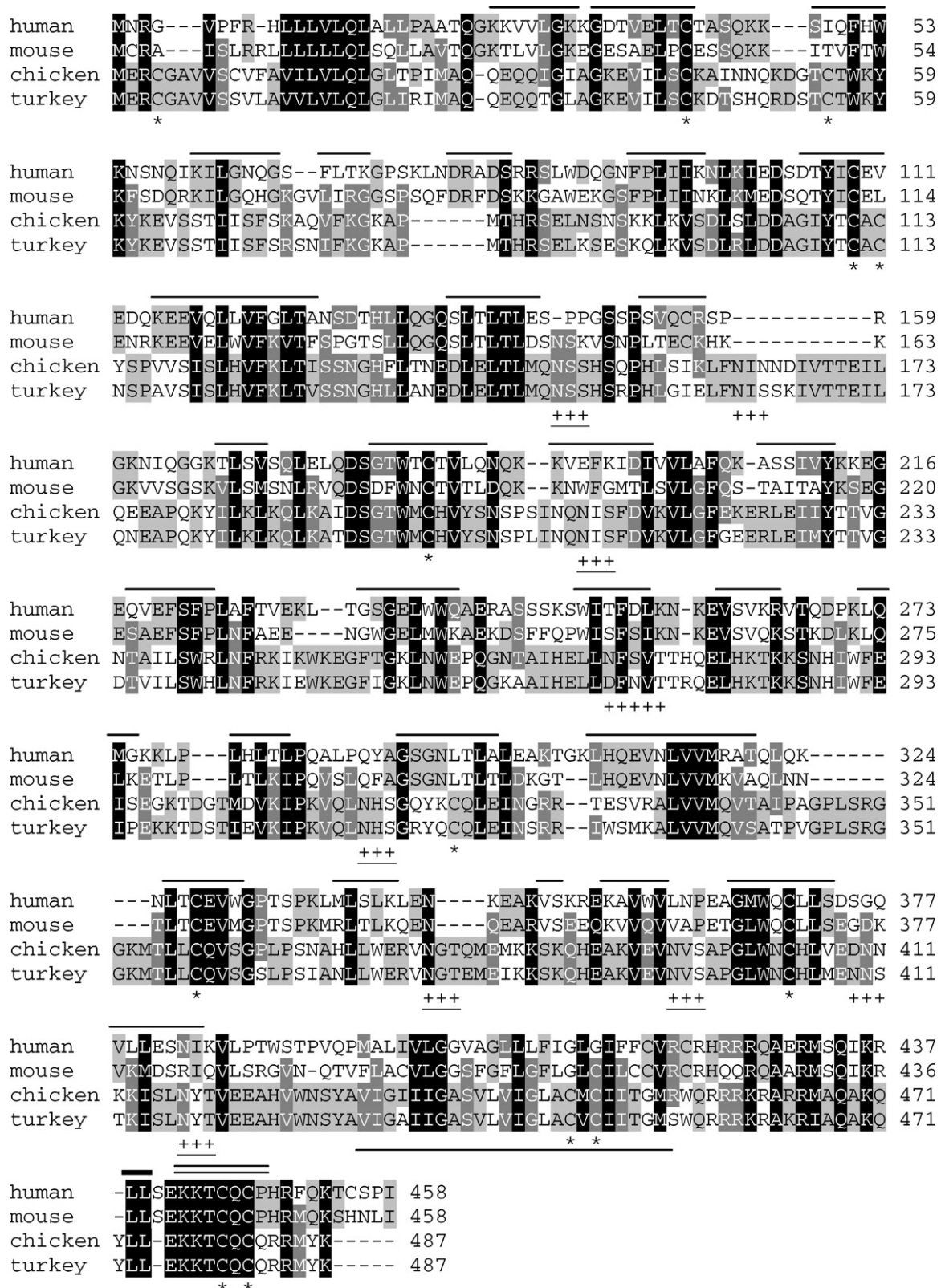


Fig. 3. Comparison of the predicted aa sequences of human, murine, chicken and turkey CD4. Shaded areas represent conservation of aa similarity; the darker the shading, the more conserved the residue across species. Dashes indicate gaps in the alignment. Conserved cysteine residues in the avian predicted proteins are indicated with asterisks. β -strands of the V domains 1–4 of human CD4 are shown overlined. The tyrosine protein kinase-binding site [18] is shown double-overlined, a di-leucine motif [20] overlined with a bar and the transmembrane domain underlined [4]. Potential N-linked glycosylation sites in the avian sequences are indicated by +++, and those conserved between both species by +++. The accession numbers of the cDNA sequences used are as follows: human, NM_000616; mouse, NM_013488; chicken, Y12012; turkey, AM884253.

3.3. Cloning of turkey CD4 cDNA

Screening of the turkey cDNA library identified two positive clones for CD4, which were sequenced with plasmid-specific and insert-

specific primers. A full length predicted protein coding sequence was obtained, along with 145 nt of 5' UTR and 166 nt of 3' UTR.

Sequence comparisons (Fig. 3) showed that sequence identity between turkey and human CD4 was 44.7% and 22.4% at the nt and

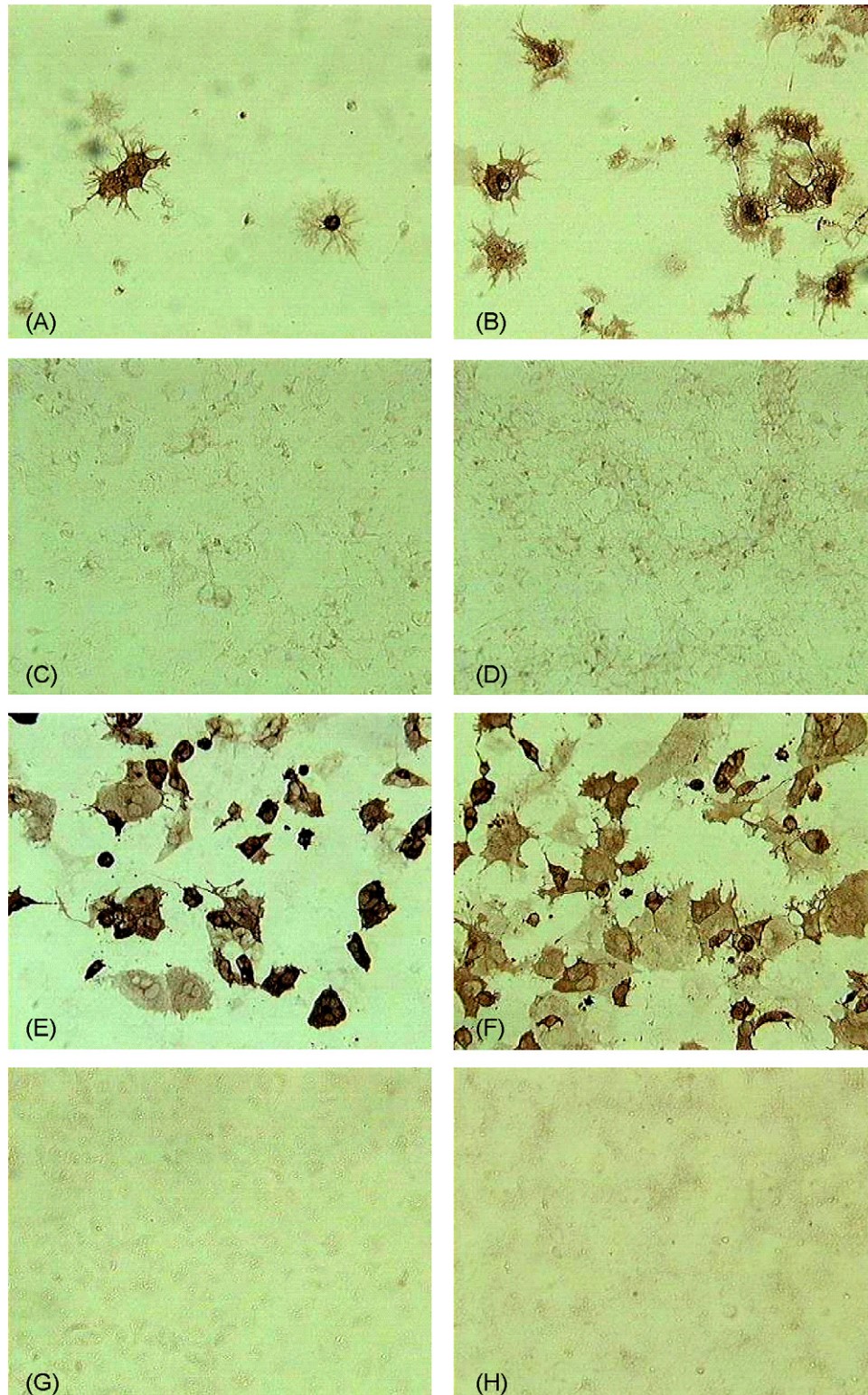


Fig. 4. Immunostaining of transfected COS-7 cells with anti-chicken CD marker monoclonal antibodies. COS-7 cells were transfected with (A) pCI-neo containing chicken CD8 α ; (B) pCI-neo containing turkey CD8 α ; (C) pCI-neo alone; (D) untransfected; (E) pCI-neo containing chicken CD28; (F) pCI-neo containing turkey CD28; (G) pCI-neo alone; (H) untransfected. (A–D) COS-7 cells stained with the anti-chicken CD8 α monoclonal antibody, 11–39. (E–H) COS-7 cells stained with the anti-chicken CD28 monoclonal antibody, AV7. Magnification 250 \times .

aa levels, respectively. Turkey and murine CD4 shared 46.7% and 22.7% nt and aa identity, respectively (Fig. 3). Identity between chicken and turkey CD4 was 91.3% at the nt and 84.2% at the aa level (77/486 differences), respectively.

There are 14 cysteines in chicken CD4. All were conserved between chicken and turkey, except for a single cysteine at position 10 in chicken CD4 which was absent in the turkey. Only seven cysteines were conserved between the avian and mammalian CD4 molecules. Like mammalian CD4, the chicken CD4 extracellular region consists of four Ig-like domains [19]. While aa replacements between chicken and turkey CD4 appeared randomly distributed across the molecule and within the predicted β -strands of the V domains, only two replacements occurred in the transmembrane region between residues 431–454. After T cell activation, CD4 is internalized [20]. In mammals, this requires the phosphorylation of two serine residues associated with a dileucine motif [21] in the cytoplasmic tail. Both chicken and turkey CD4 had the conserved di-leucine motif, although they lacked the serine residues. Two nearby tyrosine residues may be used in the chicken [19], and these were both conserved in the turkey. The tyrosine protein kinase-binding site [18] was completely conserved between the chicken and turkey. There were seven and nine potential N-linked glycosylation sites in the chicken and turkey, respectively, and six of them were conserved between the two species. The turkey CD4 sequence was submitted to Ensembl with Acc. no. AM884253.

3.4. Anti-chicken CD8 α and CD28 mAb specifically cross-react with turkey CD8 α and CD28, respectively

COS-7 cells were either transfected with pCI-neo containing turkey or chicken CD8 α , empty vector alone or untransfected. The resulting transfected or untransfected cells were then stained with an anti-chicken CD8 α mAb, 11–39 [22] (Fig. 4). COS-7 cells expressing either chicken or turkey CD8 α stained strongly with anti-chicken CD8 α .

Similarly, COS-7 cells were either transfected with pCI-neo containing turkey or chicken CD28, empty vector alone or untransfected. The resulting transfected or untransfected cells were stained with an anti-chicken CD28 mAb, AV7 [10] (Fig. 4). COS-7 cells expressing either chicken or turkey CD28 stained strongly with anti-chicken CD28.

4. Discussion

Turkeys and chickens are phylogenetically closely related, both being members of the order *Galliformes*. It is therefore to be expected that some anti-chicken mAbs will cross-react with the equivalent turkey antigens, as has been demonstrated by several groups [13–15]. In our studies, where cross-reactivity was observed, amplification of the gene encoding the turkey orthologue of the chicken antigen was attempted by RT-PCR and Genome Walking [16]. Using this approach, partial cDNA sequences of turkey CD4, CD8 α and CD28 were previously characterized [16]. In this study, these sequences were used as probes to screen a novel turkey cDNA library. The resulting clones were sequenced and full-length coding sequences of turkey CD4, CD8 α and CD28 were identified. In each case the predicted turkey proteins showed very high aa identity to their chicken orthologues, suggesting both that the molecules would have conserved functions in the two species, and confirming that reagents to the chicken CD markers have the potential to cross-react with their turkey orthologues.

The functions of chicken and turkey CD4 are likely to be conserved. Turkey CD4 has more potential N-glycosylation sites than chicken CD4. Thirteen of fourteen cysteines in chicken CD4

are conserved in turkey CD4. All of the aa residues thought to play a role in internalization of CD4 in the chicken are conserved in the turkey molecule. Finally, the tyrosine protein kinase-binding site, crucial for downstream signalling after CD4 engagement and therefore the optimum response of CD4 T cells to antigen, is completely conserved between both species.

Turkey and chicken CD8 α both have ten conserved cysteines, and the tyrosine protein kinase-binding site is completely conserved also. Two regions in human CD8 α are important in binding the α 3 domain of MHC class I, aa 47–56 and 76–81 [23]. In human CD8 α , if some of these residues are changed for acidic residues, binding to MHC class I is abolished [23]. Although there are several aa differences between turkey and chicken CD8 α in these regions, none of the substitutions are for acidic residues. Again, this suggests that the functions of turkey and chicken CD8 α are conserved.

The six potential N-linked glycosylation sites, the four cysteine residues, and the MYPPPY motif (thought to be critical in binding of CD28 and CTLA-4 to their ligands, the B7 family) in chicken CD28 are all conserved in turkey CD28, again suggesting conservation of biological function.

The large panel of reagents now available in the chicken's "immunological toolbox" provides potential tools to investigate the turkey's immune response. However, cross-reactivity of anti-chicken CD marker mAb with turkey cells is variable. Of several anti-chicken CD8 α mAb, only two cross-react with turkey CD8 α through FACS analysis (3–298 and 11–39) [14,24]. We recently used the anti-chicken CD8 α mAb 11–39 to characterize changes in CD8 T cells in the footpads of turkeys suffering from footpad dermatitis [15]. This study has shown that this cross-reactivity was specific for turkey CD8 α , reinforcing our conclusions [15] and similarly that AV7, an anti-chicken CD28 mAb, specifically cross-reacted with turkey CD28. These mAb (11–39 and AV7) can therefore be used with confidence to study cells expressing CD8 α or CD28 in the turkey and are tools to study the immune response to any pathogen of turkeys. For example, we have used these mAbs to investigate the immune response of turkeys to infection with the obligate extracellular protozoan parasite, *Histomonas meleagridis* (Powell et al., submitted). Further, the cDNA sequences for turkey CD4, CD8 α and CD28 can be used to develop real-time quantitative RT-PCR assays to measure expression of these molecules at the mRNA level in turkey tissues and cells.

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